Blood circulating tumor DNA for non-invasive genotyping of colon cancer patients

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Abstract

Most solid tumors, including colorectal cancers, shed cell-free DNA (ctDNA) in the blood. ctDNA can be analyzed to generate molecular profiles which capture the heterogeneity of the disease more comprehensively then tumor tissue biopsies. This approach commonly called 'liquid biopsy' can be applied to monitor response to therapy, to assess minimal residual disease and to uncover the emergence of drug resistance. This review will discuss ctDNA applications in the clinical management of colorectal cancer patients and will provide perspective on future development of utilizing body.

Introduction

With a global incidence of over one million cases and a disease-specific mortality of about 33%, colorectal cancer (CRC) is a major health burden (Haggar and Boushey, 2009; Siegel et al., 2014). CRC usually can be cured by surgical excision if detected at any stage before distant metastasis to the liver and other organs occur. Unfortunately, about 50% of patients

have such distant metastases, either occult or detectable, at the time of diagnosis, accounting for virtually all the deaths from the disease. Approximately half of the CRC cases are diagnosed at late stages, and this reduces the treatment opportunities.

Early detection of CRC greatly improves the chances of cure. Several approaches including colonoscopy, evaluation of serum markers as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) (Vukobrat-Bijedic et al., 2013), together with Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) scans (Sun et al., 2008) are used to diagnose CRC. The use of CEA or CA19-9 is limited by the lack of sensitivity and specificity of these markers (Ludwig and Weinstein, 2005). For instance, it has been previously shown that CEA tests displays a sensitivity as low as 43% (Keesee et al., 1996). Therefore, it is mandatory to find a noninvasive biomarker that can be employed for early diagnosis, detection of recurrence and monitoring of metastatic CRC (mCRC).

From the therapeutic side, the introduction of targeted therapies has widened the treatment opportunities for patients with mCRC. For example, the humanized monoclonal antibodies cetuximab and panitumumab, which target the epithelial growth factor receptor (EGFR), are commonly used for the treatment of mCRC. However, although both agents usually achieve incremental gains greater than those of standard chemotherapy, a large fraction of patients do not receive clinical benefit from such therapies (Bardelli and Siena, 2010; De Roock et al., 2010).

Our group and others have unequivocally demonstrated that mutations of the *KRAS* gene, which occur in 35 to 40% of CRCs, and in other genes like *NRAS* and *BRAF*, as well as amplification in *ERBB2* and *MET* genes (Bardelli et al., 2013; Bardelli and Siena, 2010; Bertotti et al., 2011; De Roock et al., 2010), impair the response to anti-EGFR antibodies.

Unfortunately, and virtually in all patients, the response to EGFR targeted therapies is transient and does not last for longer than 12-18 months (Misale et al., 2014b; Van Emburgh et al., 2014). This aspect applies to most anticancer therapies, and understanding the molecular changes that occur when secondary resistance ensues is an unmet clinical need.

The assessment of the *KRAS*/*NRAS* mutational status of CRC is now commonly ascertained using formalin-fixed paraffin embedded (FFPE) tissue (Domagała et al., 2012; Ma et al., 2009). Difficulties in accessing samples from resistant tumors are among the reasons that have so far impaired research on this topic. Assessing RAS mutational status in tissue samples is quite challenging. Firstly because of the nucleic acids degradation during the fixation procedures. Moreover, tumor sampling is inherently biased and has been shown to underestimate the genomic heterogeneity within a given tumor. Ultimately, tissue analysis only provides a snapshot of the tumor lesion in a given timeframe.

Advances in genomics and proteomics now make it possible to identify ‘factors’ released from tumors in plasma and serum, as well as in other body fluids (Crowley et al., 2013; Diaz and Bardelli, 2014; Schwarzenbach et al., 2011; Siravegna and Bardelli, 2014). Previous research has demonstrated that processed blood (plasma and serum) contain free (non-cell-bound) circulating DNA (ctDNA) but the mechanisms of release into the bloodstream and the origin of the DNA are far from fully understood (Leon et al., 1977; Stroun et al., 2000). Accordingly, in principle liquid biopsies are highly useful for continuous therapeutic assessment, reducing the need of tumor tissue biopsies.

Circulating free DNA is shed from normal (healthy) and cancer cells through two main mechanisms: active release and cell death (Stroun et al., 2001; Stroun et al., 2000). The

mechanism of active release of ctDNA into bloodstream by living cells is not completely understood (Gahan et al., 2008). Shedding by dead cells occurs by two main mechanisms: apoptosis and necrosis. The first usually generates ctDNA fragments of about 145-180 bp (or multiples of these bp), which clearly corresponds to the size of the DNA wrapped around the nucleosomes (Jahr et al., 2001; Schwarzenbach et al., 2011; Stroun et al., 2001; Stroun et al., 2000). Opposite to that, necrosis may generate more irregular and longer ctDNA fragments (up to 10 kbp). Other factors, as the action of nucleases, mononucleosome breakdown, or phagocytosis (Rykova et al., 2012), could further contribute to ctDNA fragmentation in the bloodstream.

This review focuses on applications of ctDNA in the clinical practice of colorectal cancer patients (Table 1).

Applications of ctDNA for CRC detection

Many studies demonstrated how plasma ctDNA levels alone could be exploited to closely monitor CRC patients and to readily recognize individuals with high-risk of recurrence.

Frattini et al. showed how DNA levels in plasma were significantly higher in CRC patients, while they decreased during their follow-up when patients were disease-free and went up again at tumor recurrence (Frattini et al., 2005). In a prospective biomarker study, the researchers evaluated 229 patients with chemotherapy refractory mCRC from four consecutive Phase II trials and 100 healthy individuals with the aim of establishing a normal

range of cell free DNA (cfDNA) in healthy individuals while comparing it with metastatic colorectal cancer (mCRC) patients (Spindler et al., 2015). They also went on investigating the prognostic value of cfDNA and analysed the tumour-specific KRAS mutations in the plasma.

As mentioned in the introduction, the Response Evaluation Criteria In Solid Tumors (RECIST) (Eisenhauer et al., 2009), utilizes lesion size, to assess tumor burden and response to therapy. RECIST measurements are associated with serum biomarkers, such as CEA and CA19.9 in colorectal cancer (Vukobrat-Bijedic et al., 2013). ctDNA half-life is less than two hours (Diaz and Bardelli, 2014), and changes in its levels can therefore be evident days or weeks before tumor shrinkage could be assesed by imaging or in protein biomarkers. More importantly, since ctDNA is tumor specific, it reduces or abrogates false-positivity issues associated with commonly used cancer biomarkers. It has been proposed that ctDNA, especially when used in combination with CEA, represents a potentially useful tool for the diagnosis of early-stage colorectal cancer (Flamini et al., 2006). This case-control study comprised 75 healthy donors and 75 colorectal cancer patients and found that while CEA and ctDNA were not significantly correlated in either donors or patients, when taken together, and at least one of the two resulted to be positive, the sensitivity increased (88%) but the specificity remained the same of the single measurement (70.7%). Furthermore, when both were positive, specificity reached 100%. Based on these findings, this study proposed that the combination of the two markers could be a useful tool for the diagnosis of early-stage disease.

The length of DNA fragments might also be used as a marker for tumor detection since circulating DNA integrity may reflect cancer cell death. In a study by Umetani and colleagues, the DNA integrity index was calculated as the ratio of longer to shorter fragments. The study

determined a DNA Integrity Index in sera of colorectal or periampullary cancer patients (Umetani et al., 2006), using quantitative PCR (qPCR). This study showed that shorter fragments (below 150 bp) mostly reflected the total circulating fragments (ctDNA), while cancer derived fragments were longer (above 250 bp) deriving from necrotic cells. Importantly, serum DNA integrity was significantly increased even during the early stages of CRC, suggesting that it might be used for early diagnosis. Notably however, any necrotic or mechanically disrupted cells can release longer DNA fragments, therefore, patients with injuries (Lam et al., 2003), acute inflammations or heart diseases may also have high levels of serum/plasma DNA. In pre-cancerous conditions, it is unknown whether blood from patients with benign lesions such as colonic polyps contains higher DNA integrity, and further studies are needed to determine this.

Heitzer and colleagues reported the occurrence of a biphasic distribution of plasma DNA fragments in about one third of mCRC patients (Heitzer et al., 2013). They found that not all patients with progressive metastatic disease release tumor DNA into the circulation in measurable quantities. It was observed that some cancer patients with a single peak displayed higher plasma DNA levels compared to healthy controls but, as confirmed by molecular analysis, a very low frequency of mutated DNA fragments. This indicates that the released DNA contains multiple wild-type DNA sequences, which may explain the increase in total, but not mutant circulating DNA. In contrast, a biphasic distribution may indicate a different biological process since it is associated with high mutant DNA levels in plasma, and an increased number of CTCs. This phenomenon likely reflects a massive cell death, which is followed by release of DNA into the circulation.

Genotyping cancer alleles in ctDNA of CRC patients

Detection of somatic mutations in ctDNA is the first and uttermost important application as a biomarker, because of its uniqueness in differenciating tumor-derived DNA fragments from the normal ones. Several studies focused on the assessment of hotspot alterations in *RAS* and *BRAF* genes in CRC patients (Mouliere et al., 2013; Siravegna et al., 2015; Thierry et al., 2014a; Thierry et al., 2014b).

Other exciting applications of ctDNA analysis include post-surgery surveillance. In a study by Reinert et al., in 151 plasma samples from six relapsing and five non-relapsing CRC patients, ctDNA levels were quantified using droplet digital PCR and correlated to clinical findings (Reinert et al., 2015). They were able to efficiently assess disease status through time, to monitor response to surgical and therapeutic interventions, and to detect relapses months ahead as compared to conventional follow-up. In a separate study, minimal residual disease was also correlated with the presence of higher DNA levels of ctDNA in 18 mCRC after surgical resection (Diehl et al., 2008).

In a very recent study, Tie and coworkers exploited circulating tumor DNA analysis to evaluate tumour burden and predicting response to standard chemotherapy in early stage CRC patients (Tie et al., 2015). Somatic variants (cancer-exclusive mutations) detected in tissue samples obtained at diagnosis, were tracked in the blood to monitor tumor burden non-invasively. By sequencing a panel of 15 genes frequently altered in CRC, at least one mutation was detected in 98% of the tumor tissue samples analyzed. The results showed a ctDNA detection sensitivity of 92% in the baseline samples, and ctDNA quantification

correlated closely with tumor lesion sizes as assessed by CT-scan. Moreover, the report highlighted how mutation levels in blood can anticipate response to therapy (typically after 8 to 12 weeks after treatment initiation), later confirmed by standard RECIST criteria. The study also highlighted a trend between ctDNA changes and progression free survival (PFS). These data highlight how early changes in mutational loads in plasma may reflect DNA release into the bloodstream and may be a used to identify the best responders.

In a landmark study by Bettegowda and colleagues (Bettegowda et al., 2014), *KRAS* mutant fragments were detected in the blood of patients with *KRAS*-mutant colorectal tumors, with high specificity (99.2%) and sensitivity (87.2%). They also correlated *KRAS* mutant levels with a shorter overall survival.

Monitoring drug resistance and clonal evolution in the blood of cancer patients

We and others previously reported that acquired resistance to EGFR-specific antibodies is associated with the emergence of RAS pathway mutations, and that these mutations can be detected in the blood of colorectal cancer patients before disease progression is clinically manifest (Diaz et al., 2012; Misale et al., 2012). We found that *KRAS* mutant alleles were evident in the blood of cetuximab treated patients 10 months before the documentation of disease progression by standard imaging. In a follow up study, our group discovered the emergence of multiple different resistance mutations in the same patient (Diaz et al., 2012; Misale et al., 2014a). Importantly, CRC patients who acquired resistance to EGFR antibodies displayed a heterogeneous pattern of mutation in *KRAS*, *NRAS*, *BRAF* and *EGFR* (Misale et al., 2014b; Van Emburgh et al., 2014). More recently, we evaluated whether ctDNA in blood-

based liquid biopsies could be used to track clonal evolution and targeted drug responses in CRC patients (Siravegna et al., 2015). *RAS* and *RAF* mutations found in the blood correlated with mutations found in the tissue in 97% of the 100 cases included in the study (Siravegna et al., 2015). Interestingly, in a subset of patients RAS pathway mutations were only detected in blood and not in the matched tumor tissue samples, suggesting that liquid biopsy could better capture the heterogeneous landscape of mutations. It is likely that ctDNA can comprehensively represent the overall picture of the disease.

Interestingly, we reported that levels of *KRAS* alleles that emerged at progression to EGFR inhibitors, declined after treatment interruption. The proportion of *KRAS*-mutated alleles dynamically increased and decreased in the presence and absence of the anti EGFR drug (Siravegna et al., 2015). Based on these findings, ctDNA analysis provided molecular evidence that clonal evolution during targeted therapies can be detected in circulating free DNA.

In a related study Speicher and colleagues performed whole genome sequencing of plasma of CRC patients treated with anti-EGFR therapy (Mohan et al., 2014). The results unveiled the presence of several copy number changes in all plasma samples from patients, including loss of the chromosomal 5q22 region harboring the APC gene and loss of chromosome arms 17p and 18q. Moreover, amplifications in known gene involved in the resistance to EGFR blockade such as *MET*, *ERBB2* and *KRAS* amplification were also detected (Mohan et al., 2014).

Conclusions

Genotyping ctDNA in blood samples can be used to identify the molecular profile of colorectal cancer and to closely follow its evolution during therapy. This approach can also be used to detect minimal residual disease after surgery and to identify actionable therapeutic targets, and uncovering mechanisms of drug resistance (Pantel and Alix-Panabières, 2013; Pantel et al., 2013; Siravegna and Bardelli, 2014). Importantly, ctDNA might also provide early warning that the patient has developed treatment-resistant disease.

Several studies have suggested that ctDNA analysis could be successfully coupled with imaging, furthermore in several instances liquid biopsies appeared to be capable in the monitoring response to anticancer drugs and potentially anticipate disease progression as compared to CT-scan evaluation. Moreover, recent work has shown that early tumor shrinkage (ETS) in the treatment of metastatic colorectal cancer correlates with longer survival (Tie et al., 2015).

We foresee that radiological approaches and ctDNA assessments could be combined to evaluate treatment schedules as a clinical standard in oncology. This could potentially imply an early switch in treatment, sparing unnecessary side effects, enhancing efficacy and minimizing costs.

It is unlikely that ctDNA would substitute RECIST in a next future, as imaging will always be necessary to visually determine the anatomic site of metastatic disease, the resectability of tumor lesions or the involvement of adjacent organs.

Ultimately, ctDNA analysis may allow a more comprehensive assessment of the molecular heterogeneity of the patient’s cancer, which also can lead to a more personalized and combinatorial treatment with targeted therapies. A most unique advantage of circulating tumor

DNA analysis is that it enables to follow tumor molecular evolution in time. ctDNA can be investigated repeatedly and non-invasively at different timepoints through therapy. As an example, real-time monitoring RAS mutations levels in ctDNA could be used to design dynamic therapeutic schedules of anti EGFR antibodies.

Prospective studies that are adequately powered for statistical analyses, and incorporate plasma collection, are now needed to definitively establish how well ctDNA recapitulates the heterogeneity of the tumour, and to what extent it reflects tumour evolution.

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Highlights:

- Colorectal tumors shed cell-free DNA (ctDNA) in the blood.
- Liquid biopsies can be used to define –non invasively- the molecular profiles of the disease.
- ctDNA analysis can be used to: monitor response to therapy, assess minimal residual disease and to uncover the emergence of drug resistance.
- Liquid, as compared to tissue biopsies, capture more comprehensively the molecular heterogeneity of colorectal cancers.
- Radiological approaches and ctDNA assessments can be succesfully combined to tailor treatments.
- Prospective studies, adequately powered for statistical analyses, are needed to definitively establish how to incorporate liquid biopsies in the management of colorectal cancer patients.

Table 1

ctDNA applications for detection and genotyping of CRC	
Reference	**Findings**
Frattini et al., 2005	DNA levels in plasma are significantly higher in CRC patients, they decrease during their follow-up and increase again at tumor recurrence.
Flamini et al., 2006	ctDNA, especially when used in combination with CEA, represents a potentially useful tool for the diagnosis of early-stage colorectal cancer.
Umetani et al., 2006	Since ctDNA integrity may reflect cancer cell death, the length of its fragments might also be used as a marker for tumor detection.
Spindler et al., 2015	Tumour-specific KRAS mutations in plasma have prognostic value.
Genotyping cancer alleles in ctDNA of CRC patients	
Reference	**Findings**
Mouliere et al., 2013 Siravegna et al., 2015 Thierry et al., 2014a Thierry et al., 2014b	Assessment of hotspot alterations in RAS and BRAF genes in CRC patients.
Reinert et al., 2015	Post-surgery surveillance: ctDNA levels are quantified using droplet digital PCR and correlated to clinical findings.
Tie et al., 2015	Tumour burden evaluation and prediction response to standard chemotherapy in early stage CRC patients.
Bettegowda et al., 2014	KRAS mutant fragments are detected in the blood of patients with KRAS-mutant colorectal tumors, with high specificity (99.2%) and sensitivity (87.2%). KRAS mutant levels also correlate with a shorter overall survival.
Monitoring drug resistance and clonal evolution in the blood of cancer patients	
Reference	**Finding**
Diaz et al., 2012 Misale et al., 2012	RAS pathway mutations associated to acquired resistance to EGFR-specific antibodies can be detected in the blood of colorectal cancer patients before disease progression is clinically manifest.
Siravegna et al., 2015	ctDNA in blood-based liquid biopsies is used to track clonal evolution and targeted drug responses in CRC patients. The proportion of KRAS-mutated alleles dynamically increased and decreased in the presence and absence of the anti EGFR drug.
Mohan et al., 2014	Whole genome sequencing of plasma of CRC patients treated with anti-EGFR therapy unveils several copy number changes, including loss of the APC chromosomal 5q22 region and amplifications in known gene involved in the resistance to EGFR blockade such as MET, ERBB2 and KRAS.